THE ENCAPSULATION OF RAT AORTIC SMOOTH MUSCLE CELLS WITHIN POROUS SCAFFOLD

A Senior Scholar Thesis

by

ALEXANDRA IACOB

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2008

Major: Biomedical Engineering

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Approved by:

Research Advisor: Mariah S. Hahn
Associate Dean for Undergraduate Research: Robert C. Webb

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ABSTRACT

The Encapsulation of Rat Aortic Smooth Muscle Cells Within Porous Scaffold
(April 2008)

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Growing rat aortic smooth muscle cells (RASMC) in vitro has posed numerous difficulties in the past. Smooth muscle cells are known to need a three-dimensional (3-D) structure, neighboring cells, space to allow for elongation and media to encourage normal behavior. Current technology in polymers presents a potential means to create a 3-D porous environment that mimics the natural habitat for RASMC. Through this experiment various methods have been employed to produce the optimal structure for the cells. Cells are encapsulated in rapidly degrading polyethylene glycol (PEG) bead-shaped gels. These beads are then encapsulated in a cross-linked PEG rectangular scaffold. In time, the beads degrade while the cells remain intact. In essence, this allows for the creation of open pores in which the cells remain. The cells then have the chance to elongate and assume natural shape and behavior. The method proposed has shown successfully that upon the beads' degradation, the cells remain intact within the PEG hydrogel scaffold.

DEDICATION

To my grandmother

Mrs. Elena Hanciu

ACKNOWLEDGEMENTS

I would like to thank my research advisor Dr. Hahn for all the help she offered me throughout the journey of this research. I would also like to thank her research graduate students Rebecca McMahon, Carolina Munoz and Dany Munoz Pinto who offered endless guidance in the laboratory.

Thanks also to the Office of Undergraduate Research for the assistance provided in correcting and finalizing this thesis.

Finally, thanks to my friends and especially my family for the support and encouragement offered throughout the research project. I would like to especially thank my sister and my life companion for all the patience and aid they gave me throughout.

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CHAPTER I

INTRODUCTION

Organ and tissue failure due to accidents or disease are an increasing concern in the medical field.[8] The number of needed transplants far outnumber the available donors.[7] One method to overcome this need is to create artificial organs or tissues. This study focuses particularly on blood vessels. The first tissue engineered artificial blood vessel was made in 1984 by mixing cultured endothelial cells, smooth muscle cells and fibroblasts integrated with a Dacron mesh.[3] The underlying paradigm of tissue engineering is that cells must be placed in a scaffold, or three dimensional (3D) context, that mimics the cells' native environment to encourage normal behavior, i.e. cell-cell interconnections, communications, etc.

Hydrogels are a group of materials that seem to have strong potential as tissue engineering scaffolds.[7] Hydrogels based on polymers are advantageous because they are similar to the natural extracellular matrix (ECM) in that they are well hydrated 3D "networks that provide a place to for cells to adhere, proliferate and differentiate".[2] Poly(ethylene glycol) (PEG) based hydrogels are one type of synthetic scaffolds frequently used in tissue engineering and drug delivery applications. [6]

This thesis follows the style of *Annals of Biomedical Engineering.*

PEG has interested many due to its biocompability, hydrophilicity, high permeability, controllable bioactivity, and resistance to protein adsorption and cell adhesion.[11, 12] The plain PEG chains are modified via acrylation to promote cross-linking of the chains, allowing for a structured gel. Unfortunately, one of the drawbacks of this hydrogel is its dense mesh structure of polymer chains that immobilize the "seeded" cells, preventing normal cell elongation and interconnection.[5] Upon placing cells within simple PEG scaffolds, the cells maintain a spherical shape since they are unable to break the dense structure and assume natural shape[2]. One approach researchers have developed to correct for this drawback of PEG hydrogels was to create a solid but hydrolytically degradable polymer mold, polymerized PEG around this mold, and then dissolve away the mold by the addition of sodium hydroxide. [4] This created a network of large pores within the scaffold. Neural progenitor cells were then implanted in the scaffold. However, the cells were not evenly distributed and were mostly concentrated on the surface.

A separate study attempted to introduce pores within PEG hydrogels by polymerizing the PEG macromers around polystyrene beads. The polystyrene beads were then dissolved by the addition of an organic solvent, leaving behind an open porous structure.[1] However, the use of a harsh organic solvent is undesirable in tissue engineering applications, and cell seeded in the resulting scaffolds are prone to uneven distribution. To overcome the immobilizing deficit of encapsulating cells directly into the PEG hydrogel and the uneven distribution of cells associated with other methods of creating

macropores within PEG hydrogels, harvested rat aortic smooth muscle cells (RAMSC) will first be encapsulated within carrier beads composed of a rapidly degradable modification of PEG.

Many polymers also have the possibility to be chemically altered, lowering their half-life drastically by the addition of lactides or glycolides at the end of the chains; [10] PEG is one such.[9] In this case, this secondary PEG is altered by adding glycolides or lactides to the end of each polymer chain prior to acrylation. The glycolide/lactide segments are hydrolytically cleavable and thus the hydrogel crosslinks degrade as these segments are cleaved. Degradable PEG bead “carriers” are then encapsulated within a plain PEG hydrogel. As the beads degrade, the cells will remain, as will an open-pore structure. The open pores should allow the cells to elongate and assume a more natural morphology. Figures 1 and 2 below briefly illustrate these few steps.

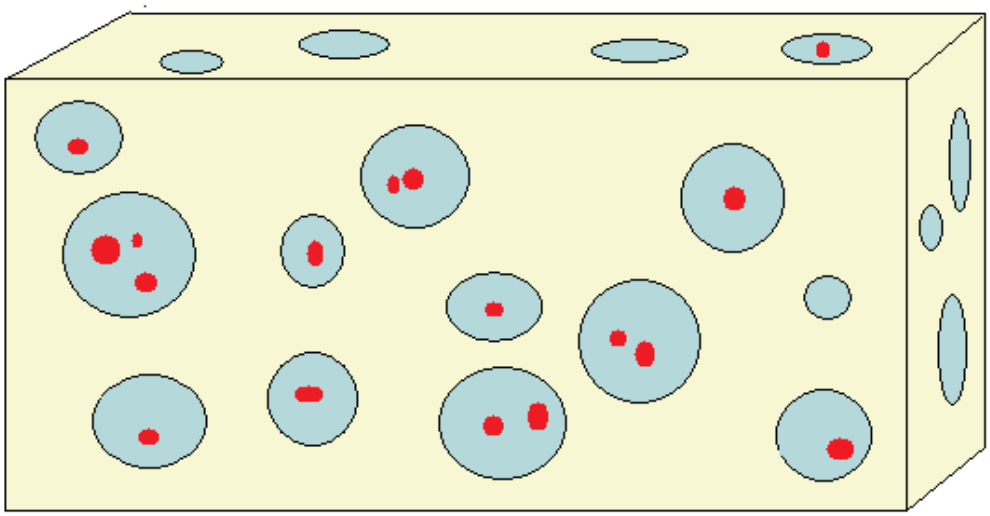

FIGURE 1. RASMC (red spheres) encapsulated in the rapidly degradable PEG beads (blue) within a PEG hydrogel (yellow).

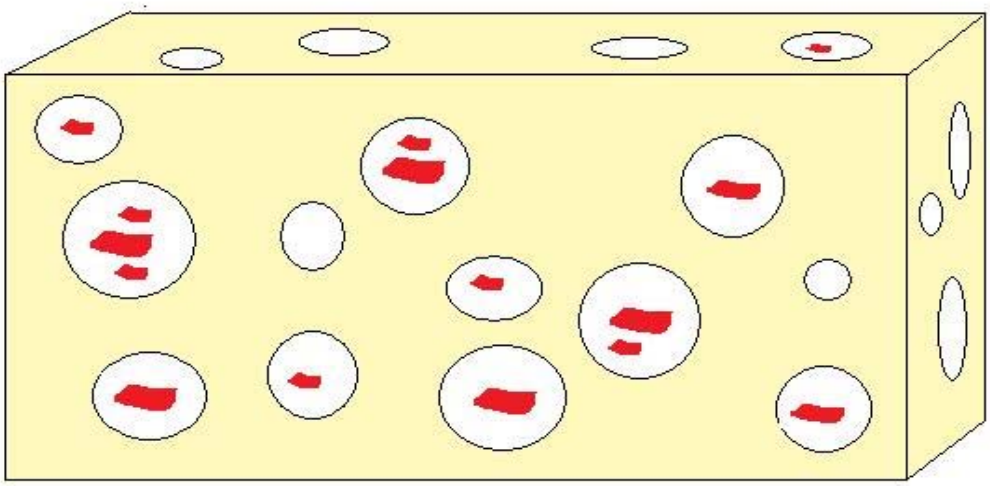

FIGURE 2. RASMC (red structures) elongated in previous beads' space within a PEG hydrogel (yellow).

This work investigates the method of generating such an optimal scaffold to encourage normal behavior.

CHAPTER II

METHODS

The methods for bead fabrication and encapsulation can be broken down in several main steps: addition of lactides or glycolides to PEG, diacrylation of PEG, forming the PEGDA beads, observating degradation of PEGDA beads, encapsulating the RASMC in the PEGDA beads, encapsulating the beads with cells in the gel to create the final scaffold, and observing the degradation of the PEGDA beads within the scaffold.

Materials and equipment

All PEG used was purchased from Fluka, argon gas from Botco, stannous octoate from Pfaltz&Bauer, Inc, dichloromethane and acryoyl chloride from Sigma, triethylamine from J.T. Baeker, $MgSO_4$ from Acros, K_2CO_3 from Fisher Scientific, and HEPES buffered saline (HBS) from HyClone. Acetophenone used in this experiment was made with 1 ml 1-vinyl-2-pyrrolidinone 99+% (purchased from Sigma-Aldrich) and 300mg 2-2-dimethoxy-2-phenyl acetophenone (purchased from TCI America). A Mercury 300 MHz NMR with field strength of 300 MHz was used along with an Ultra-Violet Products Inc. UV lamp with a wavelength of 365 nm and a Fisher-Scientific Micron Microscope

with the lens of 160/1.5. Fluorescent detection was carried out using 488/532 excitation/emission filters and an Axiovert A200 microscope.

Adding lactides or glycolides to PEG

To begin this experiment I first created the PEG glycolide. The following procedure is very sensitive to moisture, so it was conducted in a very dry and inert environment. To ensure this, all glassware was heated to 150 ^{o}C overnight and the system was evacuated with argon between each of the following steps. The purpose is to take the regular PEG chain and add an opened lactide or glycolide ring to both ends of the chain with the help of stannous octoate as a catalyst. The reaction is depicted below in Figure 3.

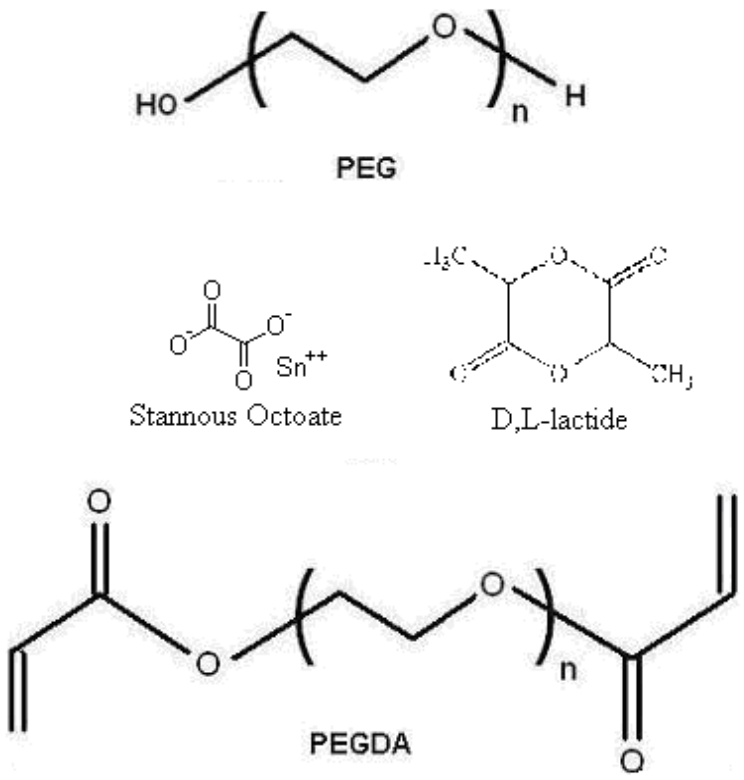

FIGURE 3. Addition of lactides to PEG followed by acrylation to create degradable PEGDA.

I started with PEG (MW 3500, Fluka) and placed it in a 250 ml round bottom flask. The flask was purged with argon in between all steps. I then added D, L – lactides or glycolides (depending on which PEG I wanted to make) in a 4:1 molar ratio of lactides/glycolides to PEG. Stannous octoate was then added in a 1:200 mass ratio to PEG. The solution was heated to 140 ^{o}C in an oil bath and the reaction was allowed to proceed for 4 h. The product of the reaction was purified in order to remove the catalyst, stannous octoate, and the unreacted lactide/glycolide by dissolving in methylene chloride

and precipitating with diethyl ether. This new PEG was analyzed using ^{13}C NMR to confirm lactide conjugation. The confirmation is achieved by a characteristic peak on the free methyl group and the peaks of the lactides/glycolide and via the quantative analysis by comparing with the areas of the peaks of the methyl protons.

Diacrylation of PEG

The PEG was placed in a 250 ml round bottom flask and dissolved in dichloromethane. The flask was then flushed with argon. Afterwards, acryoyl chloride and triethylamine were added in a 2:1 molar ratio to PEG-lactide and a 1:1 molar ratio to PEG-lactide, respectively. The reaction took place overnight, for at least 12 h. The diacrylated PEG dilactide (PEGDA) was purified by the addition of 2M K_2CO_3 followed by phase separation. This removes HCl. The organic phase was retrieved and dried with anhydrous $MgSO_4$. The product was precipitated in diethyl ether. I then dried the product thoroughly overnight and crushed it to fine powder. The powder was stored at -20 ^oC until ready to use.

Forming PEGDA beads

The PEGDA powder previously prepared was combined in a 3:10 mass ratio in a glass tube with HEPES buffered saline (HBS). The mixture was vortexed until homogeneity was obtained, for approximately 1 min, between each of the following steps. Then

acetophenone was added in a 1:50 mass ratio to the mixture, without exposing the container to any light source. This step allowed the polymer to bind to neighboring chains. Afterwards silicone oil was added in a 10:3 mass ratio. This allowed the beads to form and be completely isolated via the oil. At this point, the container was exposed to UV lamp for 10 min, allowing the beads to harden. To remove the oil, phosphate buffered solution (PBS) was added, the solution was mixed and then centrifuged at room temperature, 800g speed. The supernatant containing oil and PBS was discarded and this washing procedure was repeated twice to remove residual oil.

Observing degradation of PEGDA beads

The PEGDA beads previously prepared were simply placed in a petri dish with HBS. The beads were checked on a regular basis, at least once every 8 h. Degradation was characterized by porous surface on beads and chunks missing within the beads. When the beads had completely degraded, a film of loose milky particles floating on top was observed.

Encapsulating the RASMC in the PEGDA beads

RASMC in culture were collected and counted using a hemacytometer. The degradable PEGDA powder previously prepared was combined in a 3:10 mass ratio in a glass vial with HBS. The mixture was vortexed until homogeneity was obtained (for

approximately 1 min) between each of the following steps. The cells were added in a 5.83 million cells per each 1ml of bead volume ratio to the PEGDA solution volume. Then acetophenone was added in a 1:50 mass ratio to the mixture, without exposing the container to any light source. This step allowed the polymer to bind to neighboring chains. Afterwards silicone oil was added in a 10:3 mass ratio. This allowed the beads to form and be completely isolated via the oil. At this point, the container was exposed to UV lamp for 10 min, allowing the beads to harden. To remove the oil, PBS was added, the solution was mixed and then centrifuged at room temperature at 800g speed three times, for 6 min, 12 min, and 12 min, respectively. After each spin, the top oil layer and PBS were removed from the tube, until hardly any oil remained.

Forming the scaffold

PEGDA (MW 6k, 2 acrylations, no degradable segments) was combined in a 3:10 mass ratio in a glass tube with HBS. The mixture was vortexed until homogeneity was obtained between each of the following steps. Acetophenone was added in a 1:100 mass ratio to the mixture, without exposing the container to any light source. This step allowed the polymer to bind to neighboring chains. Afterwards the beads were added in a 19:20 mass ratio to the PEGDA solution. The solution with the beads was poured in a scaffold mold, created out of glass plates with 1.1 mm spacers, which allowed a flat 3D rectangular scaffold to form. At this point, the mold was exposed to UV lamp for 1min/side for a total of 2 min, allowing for the solution to harden. The scaffold was then

removed and analyzed with a microscope to confirm encapsulation of beads. The scaffold was then transferred to either PBS or cell culture media and maintained at 37 °C/5% CO_2.

Observing the degradation of the PEGDA beads within the scaffold

The above steps for the encapsulation of the cells in the beads and encapsulating the beads into the scaffold were followed with the single exception that eosin-Y (a fluorophor) was added along with acetophenone in a 1:1000 ratio to the bead volume. The rest of the steps were followed exactly. To view the degradation of the beads within the scaffold, the scaffold was exposed to the fluorescent setting of the microscope and eosin-Y was visible in the beads that had not degraded yet.

CHAPTER III

RESULTS

The first procedure was creating various degradable PEGDAs to test their ability to form beads and their degradation rate. The table below lists the degradable PEGDAs created with the following code: PEG MW, number of glycolides or lactides added, and the number of acrylations performed. The table also specifies whether beads were formed or not, and if so, how many days it took for the beads to degrade.

TABLE 1. PEGDAs Tested for Beads.

PEGDA	3.4k 4 glyc 6 acryl	6k 8 glyc 2 acryl	10k 8 glyc 1 acryl	6k 2 lact 1 acryl
Beads Formed	Yes	Yes	Yes	No
Days Necessary for Degradation	5	7+	3	n/a

Degradation was characterized by observing the status of the beads formed. Degradation was defined as chunks of beads missing, porous sites visible in the beads, and in the final stage loose milky appearance of beads reminisces. Figure 4 depicts the first signs of

degradation seen only after one day. The complete degradation of the beads can be seen after three days in figure 5.

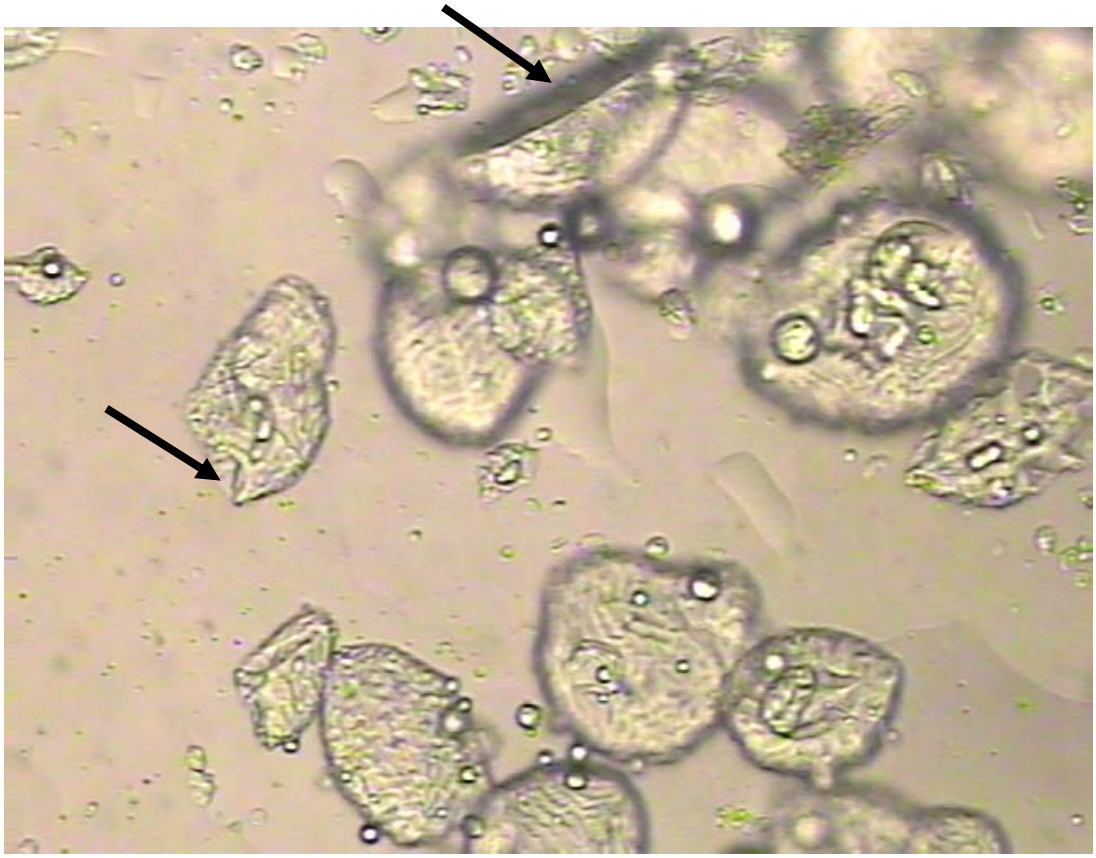

FIGURE 4. 10k 8 glyc 1 acryl PEGDA beads degradation example at one day after formation.

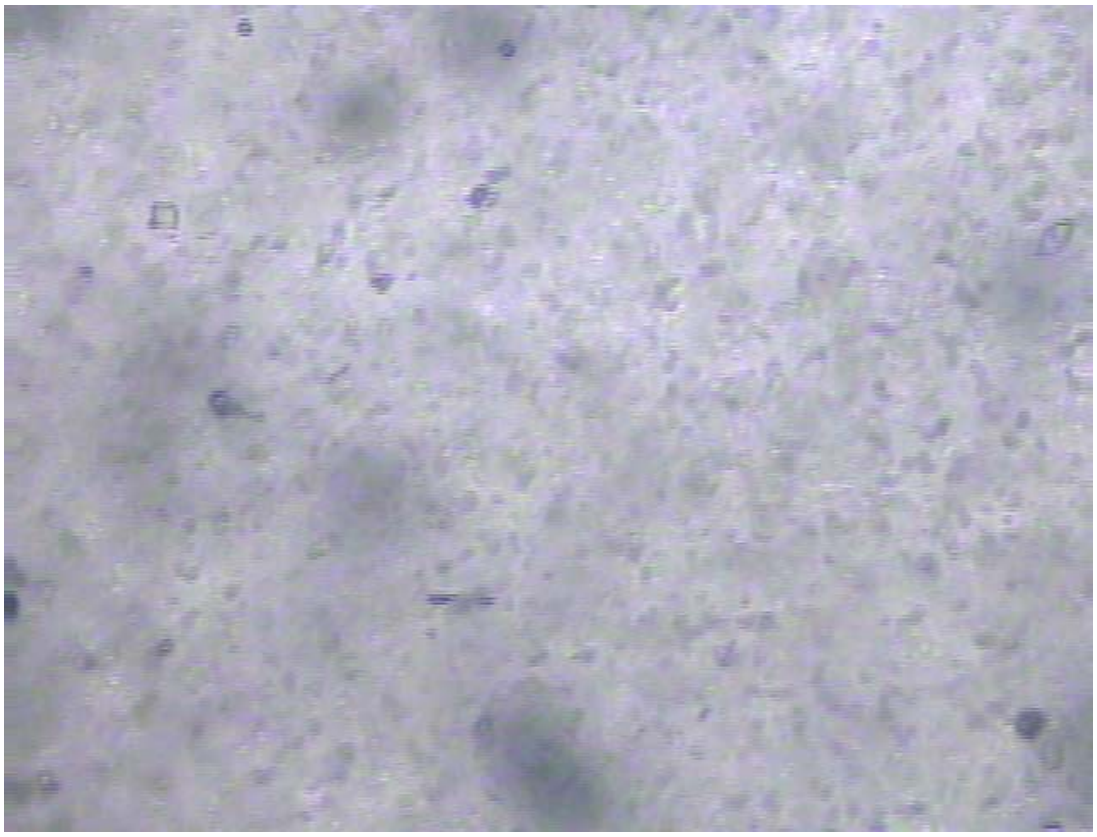

FIGURE 5. 10k 8 glyc 1 acryl PEGDA beads degradation example at three days after formation.

To verify that the PEGDA that would be used for the actual scaffold and not for the beads would not degrade considerably during the time of the experiment, we characterized its degradation in 3D rectangular scaffold form over two weeks. The following figures 6 and 7 illustrate how degradation was established.

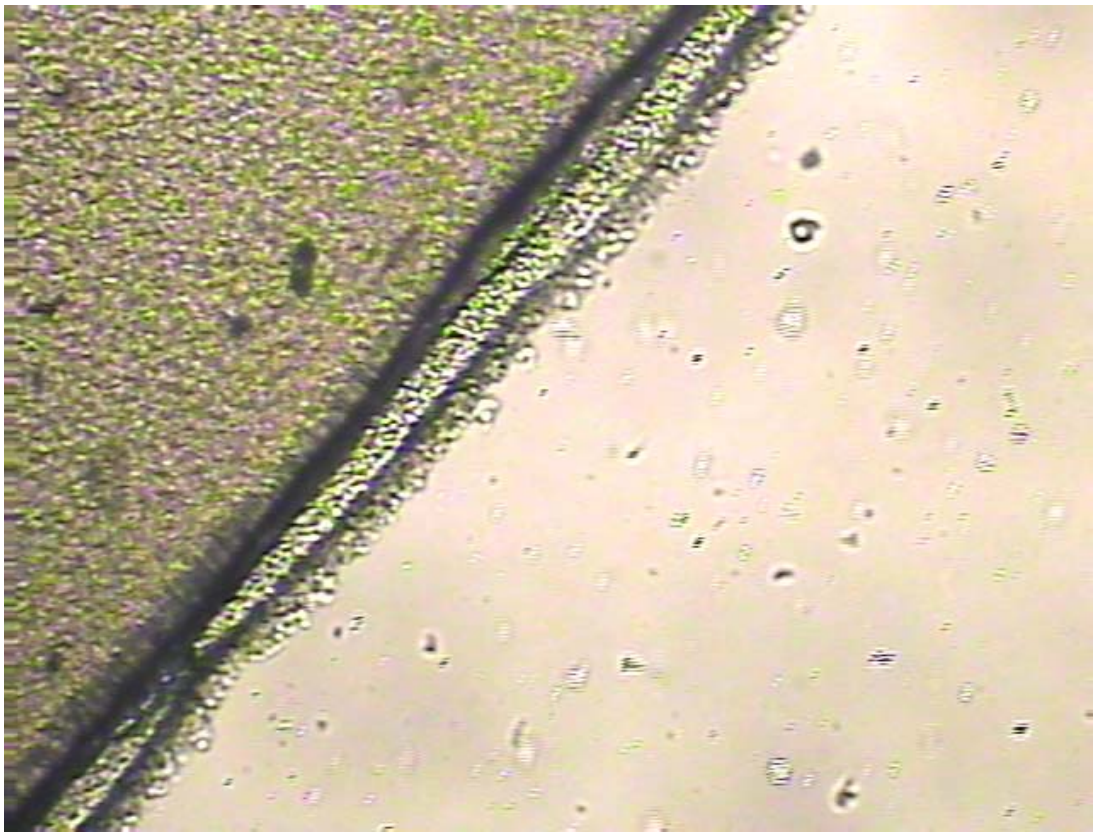

FIGURE 6. 6k 2 acryls PEGDA scaffold at formation.

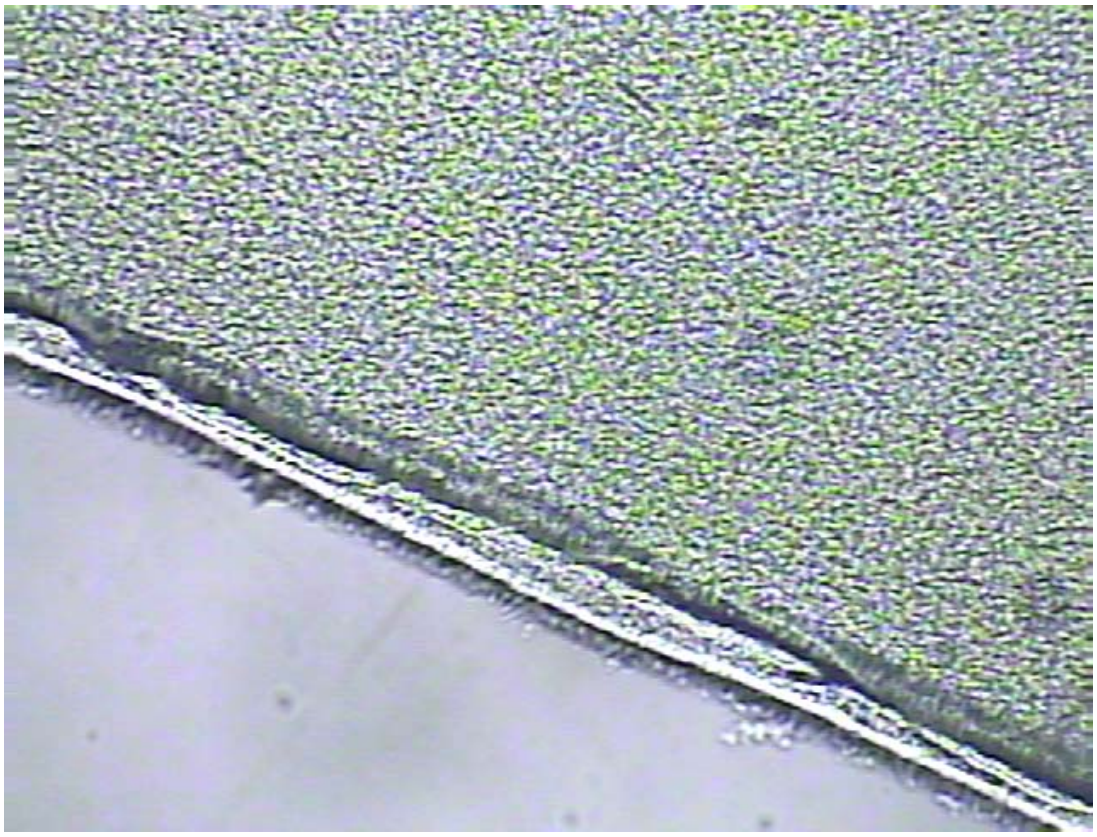

FIGURE 7. 6k 2 acryls PEGDA scaffold at five days after formation.

As seen in figure 5 and 6 no degradation was observed in 3.4K 2 acryl PEGDA: the edge of the scaffold remained intact and had no porosity appear due to degradation. This justifies its use in encapsulating the degradable beads.

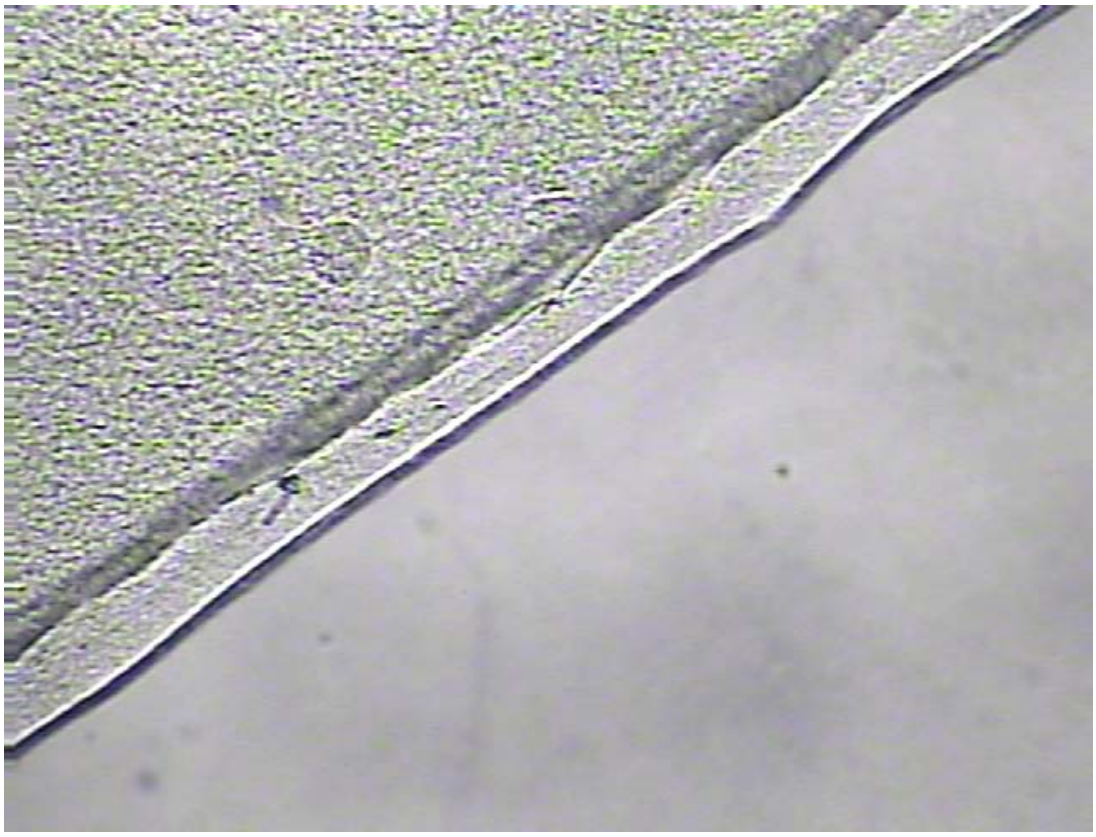

FIGURE 8. 3.4K 2 acryls PEGDA scaffold at thirteen days after formation.

After establishing which degradable PEGDA to use for the beads and that the chosen PEGDA for the scaffold is suitable since it has practically no degradation, the cells were embedded via the methods described in the previous chapter. The images portrayed in figures 8, 9, and 10 were seen through the Micron microscope. Figure 9 depicts the scaffold two days after encapsulation. At this time, the outline of the beads can still be seen (see the arrows) indicating no significant degradation yet (the cells appear as the darker red areas).

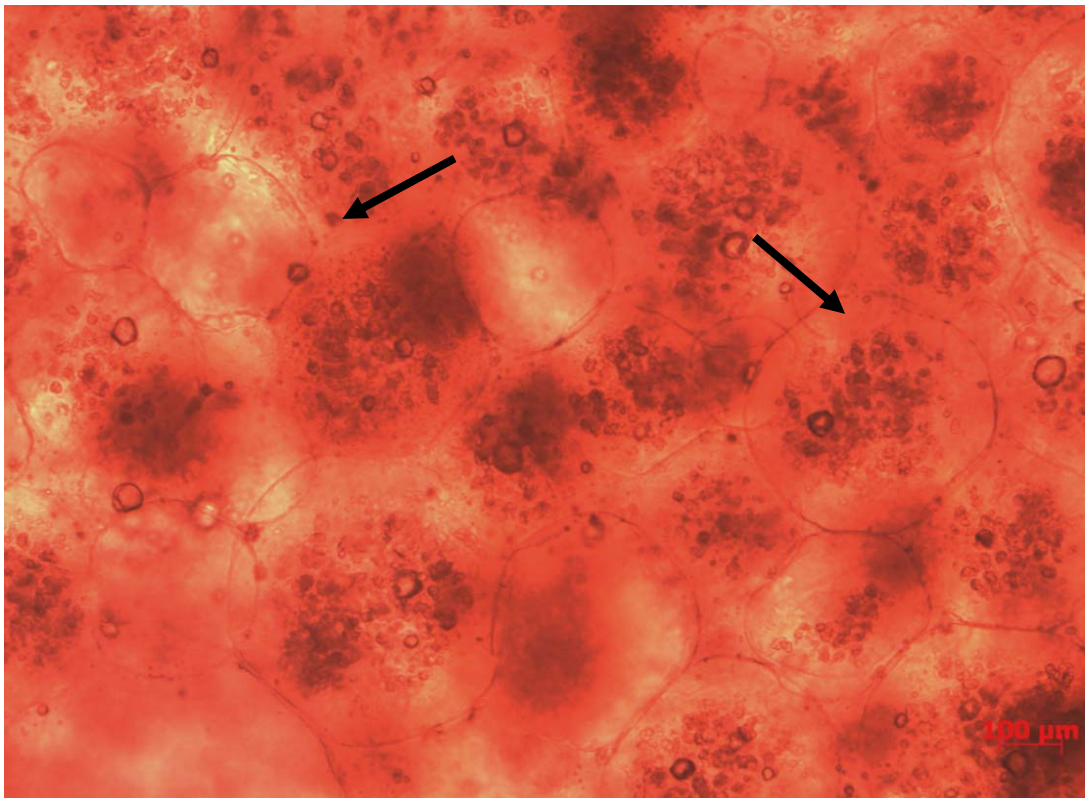

FIGURE 9. Micron microscope view of cells encapsulated within PEGDA 10k 8 glyc 1 acryl beads in PEGDA 6k two days after encapsulation.

Figures 10 and 11 show the degradation of the beads in time. Figure 10 depicts the scaffold after four days and some bead outlines can still be seen. However, some of the previously noted ones have faded, indicating degradation.

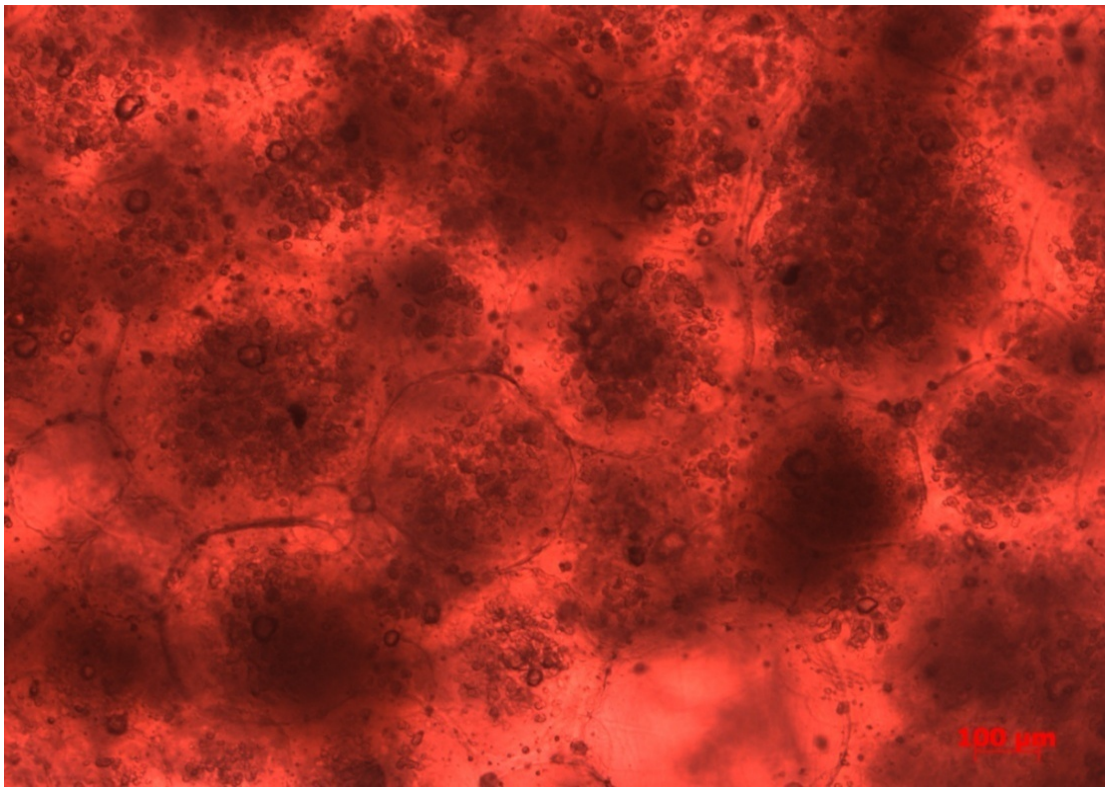

FIGURE 10. Micron microscope view of cells encapsulated within PEGDA 10k 8 glyc 1 acryl beads in PEGDA 6k four days after encapsulation.

Figure 11 depicts the scaffold at seven days after encapsulation. At this point, barely any bead outlines can be seen. Hence, the beads have mostly degraded within the scaffold and the cells seem intact.

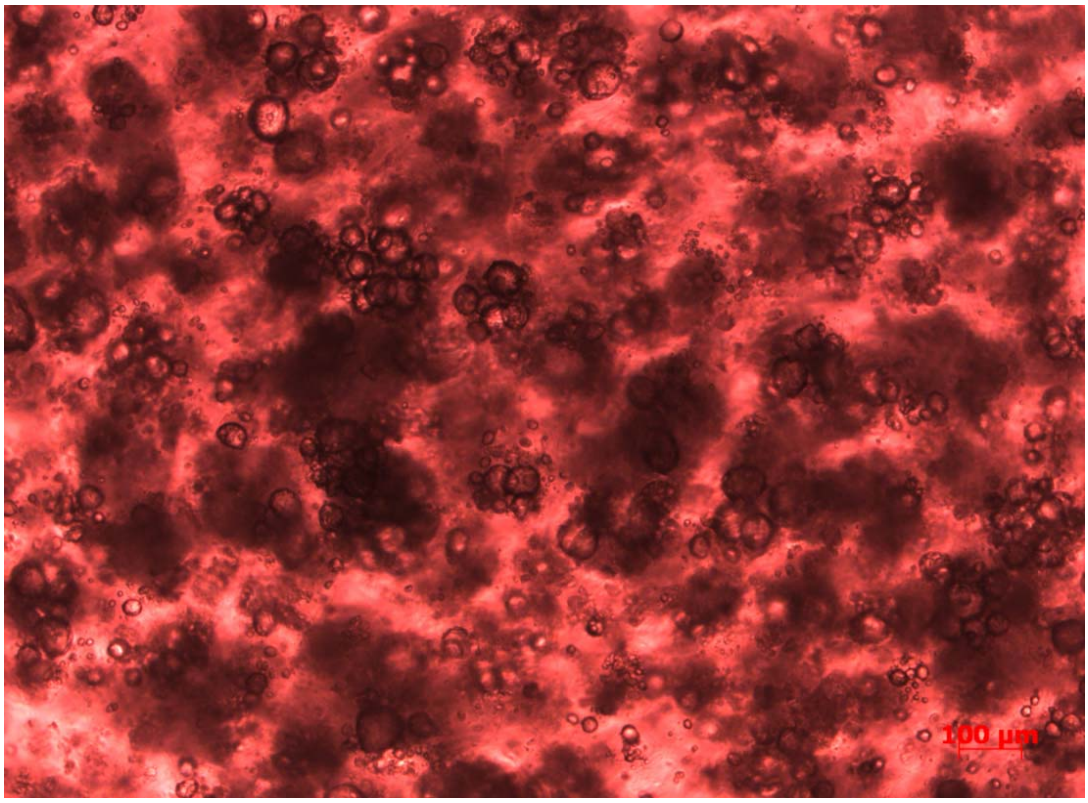

FIGURE 11. Micron microscope view of cells encapsulated within PEGDA 10k 8 glyc 1 acryl beads in PEGDA 6k seven days after encapsulation.

To further verify the method, a fluorescence technique was used. The PEGDA of the beads had eosin-Y introduced at formation allowing it to appear bright green under fluorescent light. Figure 12 shows a scaffold with only beads embedded (no cells within) which were fluorescent. This image was taken 5 days after the encapsulation. The beads closer to the edge have clearly less fluorescence than the inner beads. This finding is very important since is suggests that even though the beads do degrade (see figure 9, 10, 11) the degraded PEDGA is limited in its ability to diffuse out of the now open pore site.

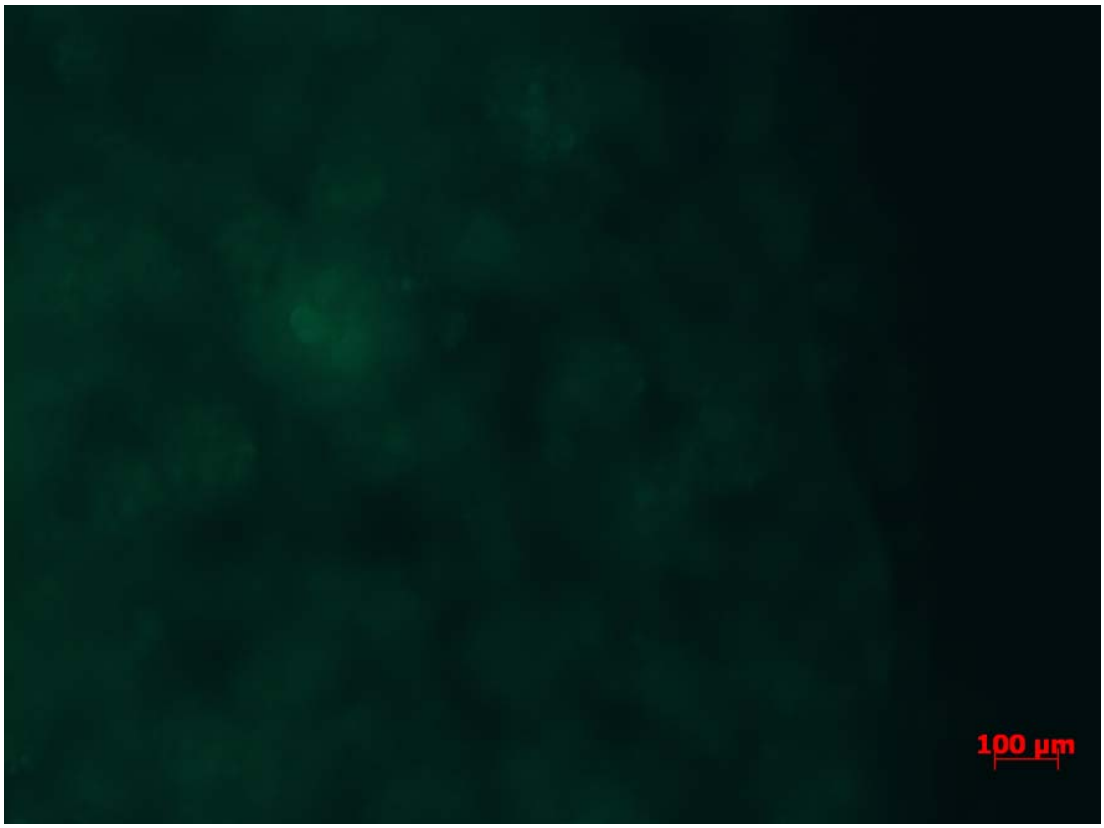

FIGURE 12. Fluorescent view of degrading beads within scaffold.

CHAPTER IV

SUMMARY AND CONCLUSIONS

The goal of this study was to find a method to encapsulate RASMC within a porous scaffold. The goal was successfully completed by first introducing the RASMC in 10k 8 glyc 1 acryl PEGDA gel, forming beads of this gel, and encapsulating these beads within 6k 2 acryl PEGDA to create the porous scaffold. The beads degraded within several days allowing more mobility for the cells. However, despite the porosity and hydrophilicity of the PEGDA scaffold, the degraded PEGDA did not leave the open pore sites.

For future studies, the scaffold should be optimized. The first problem to address is to create a scaffold in which the degraded PEGDA is able to rapidly diffuse out of the open pore sites.

A second problem to address is to find the optimal concentration of beads to scaffold and whether the bead size affects the cell behavior. This could be done by implememting various pore densities and pore sizes within the scaffold and then histologically analyzing the cells' interconnections and behavior.

A third concept would be to move from the flat 3D scaffold to a tubular scaffold. Hopefully the cells would bend with the tubular structure and thus mimic a vascular vessel. Further investigation into the method presented in this study would answer the above questions.

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